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(54) Title: NON-A, NON-B HEPATITIS VIRUS

(57) Abstract

Newly discovered particles have been found associated with non-A, non-B hepatitis. The first particle resembles a togavirus. It has a diameter of 50-60 nm with a discrete, spherical core of about 40 nm in diameter and loses its infectivity for tissue culture upon heating at 25°C in aqueous suspension or by exposure to ether. The second particle resembles an enterovirus. It is spherical, having a diameter of about 27 nm, exhibits a buoyant density of 1.36 in isopycnic density-gradient centrifugation with cesium chloride, loses its infectivity only upon prolonged heating at high temperatures and is not sensitive to ether. This particle does not have an envelope. Both particles may be cultured *in vitro* or recovered from body fluids or tissues for use in immunoassays and vaccines. The immunoassays may be employed to detect the particles, particle antigens or antiboides to the particles.

NON-A, NON-B HEPATITIS VIRUS

Background of the Invention

This invention relates to the detection and prevention of infection by newly discovered etiological agents for non-A, non-B (NANB) hepatitis. In particular this invention is concerned with making vaccines and conducting immunoassays for these agents or their antibodies.

NANB hepatitis is defined as clinical hepatitis which cannot be attributed to infection by cytomegalovirus, Epstein-Barr virus, or hepatitis A or B.

NANB hepatitis was first identified in transfused individuals. Transmission from man to chimpanzee and serial passages in chimpanzees provided evidence that NANB hepatitis is due to an infectious agent or agents. NANB hepatitis represents up to 90% of post-transfusion hepatitis cases since hepatitis B antigen - positive blood is no longer used for transfusion. The risk of contracting NANB hepatitis after blood transfusion has been estimated to be close to 10% in the U.S. Therefore, it is important to screen the blood of potential donors to detect NANB hepatitis. Further, use of donors vaccinated against NANB hepatitis would serve to reduce the probability of transmitting serum hepatitis.

NANB hepatitis is associated with a variety of virus-like particles found in serum and tissue extracts. Hollinger et al., in "Proc. Second Symposium on Viral Hepatitis" (San Francisco), p. 699, (1978) report the previous work of others in locating 20-22 nm and 60-80 nm particles associated with NANB hepatitis. Other particles which have been reported to be associated with NANB hepatitis are (a) small spheres and filaments 15-25 nm in diameter, (b) 35-40 nm diameter virions which resemble the hepatitis B DANE particle, (c) a 27 nm virus-like particle identified in lots of antihemophilic factor and liver tissue from chimpanzees infected after administration of antihemophilic factor ("Morbidity and Mortality Weekly Report" 27 (21) [1978]), and (d) a 20-27 nm diameter particle identified in hepatocytes of infected animals (Shimizu et al., "Science" 205:197-200 [1979]). In addition, antigen-antibody systems not linked to a definitive particle have been identified as associated with NANB hepatitis (Kabiri et al., "Lancet", August 4, 1979, pp. 221-224; Vitvitski

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et al., "Lancet", December 15, 1979, pp. 1263-1267; Dienstag et al.,
"Lancet", June 16, 1979, pp. 1265-1267; and Shirachi et al., "Lancet"
October 21, 1978, pp. 853-856.

Objects of the Invention

5   A principal object of this invention is to locate and isolate virus
particles which are etiological agents for NANB hepatitis.

An additional object is to purify the particles without altering
their antigenic characteristics.

A further object is to prepare vaccines from the particles.

10   Another object is to elicit or harvest antibodies against the
particles for administration as therapeutic gamma globulin or for use
in immunoassays for detecting the particles.

A further object is to produce water insoluble particles and antigens
thereof, and insolubilized antibodies capable of binding the particles

15   antigens, for use in immunoassays or for affinity chromatographic puri-
fication of the particle antigens or antibodies.·

A still further object is to provide labelled, particularly enzyme
or radiolabelled, particle antigens or antibodies.

These and other objects of the invention will be apparent from

20   consideration of this specification as a whole.

Summary of the Invention

Applicants have discovered two unique viral particles which are
associated with NANB hepatitis. These particles are principally dif-

25   ferentiated on the basis of their size, although other characteristics
are helpful in classifying them.

The first particle resembles a togavirus. It is a spherical,
coated virus having an outer diameter of 50-60 nm and a core having a
diameter of about 40 nm. Heating an aqueous suspension of this virus

30   at 25°C for extensive periods or exposing the virus to ether causes
it to lose its infectivity for tissue culture. It has been recovered
from the plasma or urine of patients suffering from NANB hepatitis.
Four samples have been placed on deposit at the Atlantic Type Culture
Collection (ATCC): VR-2011, VR-2012, VR-2013 and VR-2014. This virus

35   will be referred to herein as a togavirus-like particle.

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The second particle resembles an enterovirus. This enterovirus-
like particle is spherical, having a diameter of about 27 nm; exhibits
a buoyant density of 1.36 in isopycnic density-gradient centrifugation
with cesium chloride; loses its tissue culture infectivity upon heating
5   in aqueous suspension for 3 hours at 98°C, but not after 10 hours at
56°C; it is insensitive to ether; and its genetic material is positive
to a histochemical stain for RNA. A sample has been deposited in the
ATCC as VR-2015.

The objects of this invention are achieved in part by novel composi-
10   sitions made possible by the discovery of the above deposited virus
particles as well as the parallel discovery that both may be cultured
in vitro. The compositions comprise

(a) the purified virus particles or their antigens, particularly
those substantially free of primate protein such as human serum protein;

15   (b) the viral particles rendered biologically noninfectious or
their antigens for use as a vaccine;

(c) primate immunoglobulin which is substantially free of other
primate proteins and which is capable of binding at least one antigen
of the virus particles;

20   (d) nonprimate antibody which is capable of binding an antigen
of the virus particles;

(e) the particles and their antigens or antibodies made water
insoluble substance for use in immunoassays or chromatographic separ-
ations; and

25   (f) the particles and their antigens or antibodies bound to a
detectable label for use in immunoassays.

Further, the above objects are achieved by providing methods for
making the compositions; immunoassays comprising detecting or deter-
mining an antigen of the viral particles; immunoassays comprising
30   detecting or determining antigens present in the core of the togavirus-
like particle or antibodies to the particle core; immunoassays com-
prising detecting or determining Ig capable of binding an antigen of
the viral particles; a method for recovering NANB hepatitis antigens,
comprising separating the togavirus-like NANB hepatitis virus from urine;
35   a method for detecting NANB hepatitis which comprises assaying urine
for the presence of a togavirus-like NANB hepatitis particle antigen;
and a method for propagating NANB hepatitis virus comprising culturing

the virus in a diploid cell culture.

Detailed Description of the Invention

Unless further qualified, the term "particle" henceforth refers to the above five ATCC deposits, collectively and individually, since
5 for some purposes the togavirus and enterovirus-like particles may be considered interchangeable. "Particle" is not intended to describe the physical state of the virions.

A particle antigen is defined as a substance, normally a protein, containing at least one epitopic site of one of the deposited particles.
10 Thus, "particle antigen" includes antigens which are present in particles otherwise immunologically distinct from the particles as deposited. A particle antigen may also be present on a viral fragment or in solution, in both cases being free of any other antigenic sites otherwise present on the complete deposited particles.
15 Particle antibody is defined as an antibody capable of binding a particle antigen, regardless of the origin of the antibody. Such antibody may be obtained from convalescent serum or by active immunization of humans or lower animals with particle antigen as is more fully described below. Also, either particle antibody or antigen may be
20 purified or in association with other substances, e.g. stabilizers, carriers, serum and the like, unless such substances are specifically excluded.

The deposited samples of togavirus-like particle were discovered by electronmicroscopy in the urine of four patients in the acute phase
25 of NANB hepatitis during a hepatitis epidemic in Algeria. The particle was also detected by electronmicroscopy in the urine of five other NANB hepatitis patients out of a total of ten patients whose urines were inspected for the particle. Additionally, three of the five urine samples were infectious in tissue culture. Paradoxically, none of
30 the particles could be detected by electronmicroscopy in the sera of ten acute NANB hepatitis patients. Nonetheless, the particle has been detected in the sera of NANB hepatitis-infected patients: Out of eight hemodialysis patients whose serum glutamate-pyruvate trans-aminose activity (SGPT) was elevated above normal, the serum of one
35 patient taken at the peak of SGPT activity contained the particle as revealed by electronmicroscopy. Finally, the particle has been observed

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in a sample of commercial AHF protein fraction concentrate
which had been linked to serum hepatitis infection in those
to whom it had been administered.

5   The enterovirus-like particle was obtained from the
stool of an acute phase NANB hepatitis patient. A chimpanzee
inoculated with an extract of feces of an acute hepatitis
patient developed a NANB biological hepatitis. The particle
was observed later in samples of the chimpanzee's liver.
The particles* were found to clump upon mixture with convales-
10   cent sera from 4 out of 14 NANB hepatitis patients tested,
as visualized by immune electron microscopy. Thus these two
particles are implicated in a significant proportion of
NANB hepatitis cases.

       Either particle may be cultured _in vitro_ in tissue
15   culture. Surprisingly, the enterovirus-like particle is
particularly prolific in different diploid cell types and
culture media. While tissue culture is a well-known and
conventional art for growing many viruses, hepatitis-
associated viruses have proven to be generally difficult to
20   culture _in vitro_. Although earlier reports to the contrary
do exist, Cossart (_Virus Hepatitis and its Control_, Bailliére
Tindall, London, p. 40) observed in 1977 that the viral
agent for hepatitis B had eluded all efforts to cultivate it
in vitro. Applicants' method for culturing the particles
25   has facilitated the manufacture of vaccines and immuno-
assays as will be further described below.

       The principal variables in the tissue culture method
of this invention are cell source, culture medium, temperature
and the timing for inoculation and virus harvest. Unless
30   described below to the contrary, none of these variables is
critical within the limits one skilled in the art would
ordinarily place in general on the tissue culture of a
virus. Selection and optimization of each parameter will be
within the skill of the ordinary artisan even though some
35   routine experimentation will be required. It is important
to note that applicants' principal contribution lies not so
much in the selection of conditions and reagents as in the

  * A similar enterovirus-like particle was observed in
    the stool of an acute phase NANB patient.

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discovery that these heretofore unknown particles can be
cultured _in vitro_ at all.

The cells used in the culture must be diploid cells,
but otherwise the source is not significant. Fresh cultures
5   of cells prepared in conventional fashion from adult or
fetal organs, e.g. lung, kidney or liver, may be used, but
it is more convenient and manufacturing is better controlled
by cell cultures stable to indefinite replication, i.e. cell
lines. Representative examples of suitable cell lines are
10 MRC-5 and WI-38, all of which are available from depositories.
The cells are preferably primate although other animal cells
suffice.

Any culture medium conventionally used to grow and main-
tain the susceptible cell line is satisfactory for initially
15 culturing and then sustaining the cells during viral
replication. The preferred medium is disclosed by Morgan
et al., "Proc. Soc. Biol. Med." 73:1 (1950). It is supple-
mented with antibiotics and a small proportion, usually
about from 5% to 15% by volume, of human or animal serum.
20 If human serum is used it should obviously be free of anti-
bodies to the particles. Air is a suitable atmosphere; the
culture container should be ventilated in a sterile fashion.

The cultivation temperature is lower than the biological
inactivation temperature of the particle in tissue culture,
25 readily determined by cultivating the particle over a range
of temperatures. The togavirus-like particle is biologically
inactivated at temperatures above about 25^0C upon one passage
through tissue culture, but the enterovirus-like particle is
considerably more stable. Generally, this particle may be
30 cultured through a large number of passages at up to about
40^0C, ordinarily about from $30-40^0C$. Optimal cultivation
temperatures will vary depending primarily upon the cell
source and growth medium composition.

The tissue culture is allowed to grow to confluence on
35 the surfaces of a conventional culture chamber, so as to
maximize the population of susceptible cells. Innoculation
with particles should then follow promptly, especially if a
stable cell line is not being used. An inoculum containing
about from 1×10^5 to 1×10^8 particles/ml may be used,

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ordinarily from prior culture although pathological samples
may be used so long as they are pretreated to remove sub-
stances which are toxic to the cell culture. It is not
necessary to exchange the tissue culture medium for brief
5   cultivation periods. However, waste product buildup can
interfere with cell survival and viral replication after about
2 days, so a medium exchange may be desirable.

    The culture should continue for about from 1 to 7 days
to achieve maximum particle yields. This period will vary
10 considerably and should

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be determined for each set of conditions.

Yields of the enterovirus-like particle are high, on the order of 1×10^9 particles/ml. These particles are infective for further tissue culture, and have remained so for over 20 passages. The par-
5   ticles are recovered from the culture by simply decanting and combining the spent media from the culture container and then purifying them using one or more of the procedures described below. The purified particles are preferably frozen in a medium consisting of 50% of the growth medium without serum and 50% glycerol by volume.

10   The first subculture of the togavirus-like particle will produce about from 1×10^4 to 1×10^5 particles/ml. These particles are not infective for further culture and are therefore believed to have become attenuated as a result of replication under the conditions described. As will be described below, such particles may be useful as
15   vaccines.

The particles may also be reproduced in vivo by infecting a chimpanzee with pathological material. The animal is monitored for hepatitis symptoms. Plasmapheresis can then be used to recover large quantities of infected plasma during the acute phase. Antibody can
20   also be secured from this plasma. Alternatively, the animal may be sacrificed and the liver excised and homogenized as a source of particles.

It may be desirable for a variety of reasons to purify the particles present in pathological material such as tissues, urine and blood fractions, or which are obtained through tissue culture. For example,
25   if the particle is to be treated and employed as a vaccine or in an immunoassay, there ordinarily should be as little in the way of extraneous protein contamination as possible. Thus, the particle or its antigens should be substantially free of primate proteins. The term substantially free does not mean that the particle antigen is devoid
30   of proteins present in its natural environment which are noncovalently bound or adsorbed thereto, e.g., antibodies. However, the term does mean that all but trace concentrations of the unbound proteins normally associated with the particle or particle antigen in the pathological material or tissue culture supernatant have been removed,
35   e.g., normal human serum proteins, urinary albumin and cellular proteins.

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Particle antigens are purified by first being separated
from water insoluble contaminants having greater dimensions or
different densities. Such contaminants may include animal
cell debris, e.g., from tissue culture; cellular micro-
5 organism, e.g., bacteria inurine; or chylomicra in sera.
This gross separation is generally accomplished by low speed
centrifugation or filtration, the parameters of which will
obviously vary depending upon the nature and degree of con-
tamination and can be determined by routine experimentation.
10 Filtration is preferred as an initial step in processing
large volumes of particle suspension. Ordinarily, filters
having an average pore diameter of 0.8 micron may be used to
retain gross contamination and pass the particles.

The antigens may be separated from undesired materials
15 after gross contamination is removed. Suitable techniques
include precipitation, ultrafiltration and ultracentrifugation,
all of which are capable of distinguishing particles and
particle fragments or antigens from relatively lower molecular
weight, water soluble contaminants such as proteins and salts.
20 For example, a gross filtrate such as described above may be
ultrafiltered with a membrane having a molecular weight cut-
off intermediate the approximately 50 million of the particle
and the nearly 1 million of most commonly encountered protein
contaminants. The retentate is washed with a buffered solution,
25 the washed retentate recovered and then mixed with buffers
and stabilizers such as hydroxyl compounds, e.g., sugars,
glycerol or carbohydrates; sulfhydryl compounds such as
cysteine; and proteins such as serum albumin.

It may be more convenient to precipitate the particles
30 or their large molecular weight fragments using one or more
various well-known floculating or protein precipitating agents,
particularly salting out agents or floculating polymers.
Examples include polyethylene glycol and ammonium sulfate.
The precipitating agent may be readily removed from the pre-
35 cipitate by dialysis and the particles resuspended in buffered
stabilizer solutions for further purification or use.

The concentration of polyethylene glycol to be used will
depend upon which particle is to be purified, whether or not the particles
are intact, the electrolyte constituents of the sample, the temperature,

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the molecular weight of the polyethylene glycol and the particle con-
centration. In general, polyethylene glycol of average molecular 6000
may be added to serum to a concentration of about from 10 to 20% by
weight at a temperature of about from 1° to 5°C to precipitate the

5   particles. Precipitation from urine may not require as much polyethy-
lene glycol as with serum.

The ammonium sulfate concentration to be used is dependent upon
the factors noted above for polyethylene glycol, except that the
molecular weight of the precipitant is obviously not a variable.

10   Generally, ammonium sulfate at about from 12 to 18% of saturation is
satisfactory to precipitate the particles.

Continuous ultracentrifugation is preferred for making vaccines
as it is a physical process that introduces no potential contaminants
and can be practiced economically in large scale. Other methods which

15   can be used to purify the particles or their antigens include centri-
fugal sedimentation in a sucrose or cesium chloride gradient, zone
electrophoresis, or ion exchange, gel or affinity chromatography.

Chromatography is a versatile method since it may be readily
scaled up for commercial manufacture of the particles. Gel chroma-

20   tographic systems using cross-linked dextran beads are preferred due to
the comparatively low cost. A column of suitable gel can be selected
which will permit diffusion of proteins and low molecular weight sub-
stances into the void volume of the gel beads, thereby retarding the
progress of these contaminants through the column, while allowing the

25   particles to pass through virtually unimpeded. The gel which is select-
ed will thus be a matter of routine experimentation.

Any of the above methods may be combined as desired. For example,
gel chromatography of contaminated serum on Biogel A5M and subsequent
isopycnic centrifugation in cesium chloride is effective in removing

30   human serum proteins.

If a large proportion of the particle antigens present in a sample
are particle fragments or water soluble protein antigens, then the most
expeditious technique for separating them may be affinity chromatography.
Antibodies capable of binding the particle antigens are covalently

35   linked or absorbed to an insoluble support using conventional procedures.
The insoluble antibody is placed in a column and the sample is passed

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through. Immunologically-bound antigen is washed with buffer
and then released by changing the ionic strength or pH of the
wash buffer. Generally, acid pH is effective at releasing the
bound antigen. Antigen prepared in this fashion is essentially
5 free of contaminant proteins.

The contaminating substances which may be removed from
pathological samples include cellular microorganisms, urea,
bilirubin, uric acid, albumin, mucins, electrolytes such as
calcium, iron, sodium, potassium, magnesium, chloride, phosphate,
10 ammonia and sulfate, cholesterol, fatty acids, proteins such as
albumin, α_1-globulins, α_2-globulins, B-globulins, fibrinogen,
and lipoproteins.

The particles obtained from pathological sources such as
serum or urine may be immunologically complexed with antibody,
15 but antigens essentially free of adherent antibody are desir-
able for use as reagents in immunoassays.

Such purified particles or their antigens may be made free
of primate proteins, including any particle-bound antibodies,
by tissue culture as described above but in a culture medium
20 free of undesired proteins, e.g. cells growing in a culture
medium containing no human protein. Even if the original
pathological sample is primate, after several passages through
tissue culture the initial contaminants are diluted to unde-
tectable concentrations.

25 Purified antibody to particle antigen is also useful in
immunoassays. The impure antiserum source may be the sera of
patients convalescing from a NANB hepatitis infection or an
animal which has been immunized against the particle antigens.
The immunized animals may be nonprimates, generally guinea pigs,
30 rabbits, goats or horses, which are not susceptible to hepatitis
and thus can be injected with particles which would otherwise
be infective and potentially lethal.. The antibody-containing
gamma globulin fraction may be purified by any conventional
protein fractionation procedures previously employed to separ-
35 ate gamma globulin from plasma, e.g., alcohol, polyethylene
glycol or ammonium sulfate precipitation.

The degree of antibody purity desired will depend upon the
source and intended use. Gamma globulin for therapeutic or
prophylactic administration in the treatment or prevention of

NANB hepatitis should be of human origin and should be of as high degree of purity as commerically feasible. Usually, this at least entails removing the other desirable fractions of human plasma, e.g., antihemophilic factor or prothrombin

5 complex, from the donor plasma.

Vaccines can be made from either the togavirus-like or enterovirus-like particles, fragments and water soluble antigens thereof. Intact particles are preferred because they necessarily comprise the complete spectrum of antigenic determinants

10 and will confer the highest degree of immunity. It is also desirable that the vaccines be free of non-human proteins from such sources as the nutrient medium, cell lines or tissues in which the virus is cultured. Intact particles meeting this requirement may be made by culturing the viruses on human cells

15 in a medium which contains only human protein or a protein substitute such as synthetic, low molecular weight peptides.

Intact particles for use as vaccines must be attenuated or inactivated to eliminate biological infectivity. This may be accomplished by treatment with an agent such as formalin

20 that will not significantly affect the immunogenic or antigenic characteristics of the preparation. A satisfactory procedure entails adding formalin to the particle-containing solution or suspension and incubating under conditions which will destroy the infectivity of the composition, usually incubation at about

25 from 25^0 to 40^0C for about 1 to 5 days, or by heating under the inactivation conditions for the two particles as described above. Formalin is removed or neutralized after inactivation, although a bacteriostatically effective amount of formalin, e.g., 10 ug/ml, may be left in the vaccine. The viral particle count

30 is adjusted to about from 1×10^7 to 1×10^{11} by the addition of a physiologically acceptable carrier such as saline, by removal of excess diluent through ultrafiltration, or by lyophilization and reconstitution to the desired concentration. An adjuvant may also be added.

35 Vaccination may be conducted in conventional fashion, for example by subcutaneous administration of 1 ml amounts of the vaccine at one month intervals for a total of three immunizations. A booster at one year may be required.

Any of the immunoassay methods heretofore disclosed in the art may be used to determine particle antigens or antibodies. The following disclosure is generally expressed in terms of determining antigens. However, antibodies may be determined using the same methods.

5   The analytical methods described herein for assaying particle antigens or antibodies should not be construed as requiring the determination of all of the particle antigens or epitopic sites. Similarly, it is not necessary to assay for all of the antibodies which can bind the population of epitopic sites presented by the particle. The reason

10   for this is that mutations and varieties of viruses are frequently found in nature. Thus, some antibodies originally obtained by immunizing an animal against particles may not bind, or may bind very weakly to related NANB hepatitis strains. Fortunately, either of the viral particles described herein contain so many epitopic sites that the failure

15   of some antibodies to bind variant sites will not significantly affect immunoassay effectiveness; a sufficient proportion of the antibody population will remain capable of binding antigens common to both the immunizing and test viruses. It is even possible to base the immuno-assay upon two or, in some cases, one epitopic site. The antibody

20   used in detecting particle antigens may be raised by immunization against applicants' deposited particles, against strains newly isolated from nature containing cross-reacting antigens, or against a fragment of those strains or new isolate. Similarly, particle antigens used in immunoassays for antibody may be derived from the

25   deposited particles, a new isolate of a virus containing a cross-reacting antigen or a fragment of either the deposited particles or new isolate. A cross-reacting particle antigen is one which binds an antibody produced against an antigen of another virus with at least about 40% of the avidity as the antibody binds the antigen of

30   the immunizing virus.

The togavirus-like particle is unique in that its antigens may be divided into two groups, surface and core antigens. Surface

antigens are those which are expressed by the intact particle.
These antigens are generally those viral proteins which are exposed
to the environment on the surface of the lipid-containing envelope.
They are the antigens of greatest interest in diagnosis and immuniza-

5   tion because the intact particle is most likely to be infective
and is generally found in early states of the disease.

Incomplete particles may consist of the 40 nm diameter core only.
Such particle fragments may arise from stress which destroys the en-
velope or as a result of incomplete synthesis of the particle. The

10   core antigens may be determined in the same fashion as the surface
antigens. Core antigen may be prepared from intact particles by
contacting the particles with a detergent such as sodium dodecyl sul-
fonate which ruptures the envelope. The core may be readily separated
from the residual disrupted envelope by conventional procedures such

15   as ultracentrifugation or precipitation. The separated core or envelope
antigens may be labelled or insolubilized as described below or used
as immunogens to prepare antibodies.

Antibodies to either or both of the population of surface or core
antigens may be assayed. Such antibodies will generally fall into two

20   diagnostically interesting classes, immune globulins G (IgG) and M
(IgM). It is preferred to assay IgM as it is the antibody class which
first appears during the course of infection, when IgG synthesis may
not yet have been initiated. However, IgG assays may be material for
governmental regulation because this particular fraction is detectable

25   for many months after infection, thus demonstrating that a potential
blood donor has at one time been exposed to the particle antigen even
though the donor may no longer be infectious.

Many suitable methods for determining IgG or IgM exist. For example,
the IgG and IgM fractions of a test sample may first be separated by

30   conventional procedures, most conveniently by specifically absorbing
either fraction to an insoluble surface. For example, insoluble
anti-IgG or anti-IgM antibodies may be produced in a first animal
species against the IgG or IgM of another animal species. Alternatively,
Boyle et al., ("J. Immunological Methods" 32 (1):51-8 [1980]) disclose

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that immobilized staphylococcal Protein A and concanavalin A will
bind IgG or IgM antibodies, respectively. Thus, any of the foregoing
specific adsorbents may be employed to separate IgG from IgM. Then
a conventional immunoassay may be used to determine the separated
5   globulin fraction.

Either heterogeneous or homogeneous immunoassay techniques are
satisfactory for determining particle antigens or antibodies.
Heterogeneous assays are preferred in determining intact particles or
large antigenic fragments. Heterogeneous methods all include a step
10   of separating a solid phase with bound analyte from an aqueous
phase. In such assays an antigen or antibody is first
insolubilized, as for example by adsorption onto plastic
beads or the inner wall of a plastic test tube, by covalent linkage
to an insoluble substance or by binding to a specific binding partner
15   already so adsorbed by linking.

One heterogeneous assay is the "competitive" method. Here, un-
labelled sample analyte, e.g. antigen or antibody, and labelled
analyte analogue compete for a limited number of insoluble sites capable
of binding the analyte. Then the insoluble, bound analyte is separated
20   from the free analyte and the distribution of labelled analyte deter-
mined. This method is preferred for the assay of particle antibodies.
In such a case, the particle antigen is insolubilized, and labelled and
sample particle antibody compete for limited antigen sites.

The "sandwich" heterogeneous method is also suitable for deter-
25   mining particle antigen or antibody, preferably for determining particle
antigen as intact particles or large fragments. The method comprises
adsorbing the sample analyte onto insoluble binding partner, removing
the residual sample, adsorbing labelled binding partner onto the now
insoluble analyte, removing excess labelled binding partner and deter-
30   mining the distribution of label, usually by assaying the amount of
label bound to the solid phase. The insoluble binding partner is pre-
ferably particle antibody. A variant of this method comprises directly
adsorbing antigen from the sample onto a nonspecific adsorbent, e.g.,
a polyolefin, followed by labelled particle antibody or, sequentially,
35   particle antibody and labelled antigen.

Another suitable technique is sequential saturation. As with
the sandwich method, a surplus of insoluble binding partner to analyte
is used to bind the sample analyte, if any. However, rather than next
contacting the insoluble analyte with labelled binding partner, label-
5 led analyte analogue is added to occupy the remaining insoluble binding
sites. Unbound labelled analyte is removed and the label content of one
of the separated phases is determined.

Homogeneous immunoassay methods may also be employed to advantage.
These methods have in common the elimination of the above-described
10 phase separation; the complete determination can be conducted entirely
with water soluble reagents. One such method for determining particle
antigen comprises labelling particle antigen with an enzyme to form a
conjugate, following by admixing the conjugate, sample and antibody
to the antigen, and finally determining the change in enzyme
15 activity brought on by binding of the conjugate with antibody not
occupied by antigen in the sample (U.S. Patent 3,817,837, incorporated
herein by reference).

Another homogeneous assay, which may also be used in a heterogen-
eous mode, is disclosed in U.S. Patent 3,935,074, also incorporated
20 by reference. In an embodiment of this method for determining an
antigen, a conjugate is formed by covalently linking the antigen with
a detector ligand. Antibodies to both the antigen and detector ligand
are then mixed with the conjugate and sample. Then the usual practice
is to measure the residual, unbound antibody to the detector ligand;
25 it will be found in inverse relation to the amount of particle antigen.

Other suitable assay systems are disclosed in U.S. Patents 4,006,-
360; 4,134,792 and 3,996,345; Yorde et al., "Clinical Chemistry" 22
(8): 1372-1377 (1976) and U.S. Patent application serial number 572,008,
all of which are incorporated by reference.

30 The labels to be used in the above methods are well-known. Examples
include radioisotopes, enzymes, coenzymes, enzyme modulators, stable
free radicals, and fluorescent and luminescent groups. Preferred
labels are radioisotopes, particularly ^{125}I, or enzymes.

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The particle antigens or antibodies may be labelled with radio-iodine in any conventional manner. Suitable methods use chloramine-T or lactoperoxidase, e.g., as disclosed by Dermody et al., "Clinical Chemistry" 25 (6):989-995 (1979) or

5 Parsons et al., "Analytical Bio-chemistry" 95:568-574 (1979), externally radio-iodinated small molecules such as (^{125}I) iodohydroxyphenyl propionate-N-hydroxysuccinimate ester (Bolton et al., "Biochem. Journal" 133:529-533 [1973]), (^{125}I) diiodofluorescein isothiocyanate (Gabel et al., "Analytical

10 Biochemistry" 86:396-406 [1973]), tertiary-butoxycarbonyl-L-(^{125}I) iodotyrosine-N-hydroxysuccinimide ester (Assoian et al., "Analytical Bio-chemistry" 103:70-76 [1980]), or ICl (Montelaro et al., "Analytical Biochemistry" 99:92-96 [1979]). While the chloramine-T method is preferred for radiolabelling

15 antibody or the core of the togavirus-like particle, the technique disclosed by Montelaro et al. is preferred for labelling the surface of either particle.

The invention will be more fully understood by reference to the following contemplated examples.

20 Example 1

Urine collected from a hospitalized patient diagnosed to be in the acute phase of NANB hepatitis is first centrifuged at 8500 X g for 10 min. 1 ml of the supernatant is layered in a 4 ml gradient of sucrose (10-20%), then ultracentrifuged

25 for 3 hours at 240,000 X g using an SW 50 rotor in a Beckman L5.65B centrifuge. The pellet is resuspended in 50 to 100 μl of 0.01 M tris saline buffer at pH 7.2. The specimen is applied. to carbon grids, negatively stained with 1% uranyl acetate and examined in a Jeol 100 electronmicroscope. A serum sample

30 from an acute phase haemodialysate patient is treated in the same manner. Toga-virus-like particles are observed in both samples. In both instances the particles had an outer diameter of 54-57 nm and a core of about 40 nm.-

Example 2

35 A urine sample in which the togavirus-like particles described in Example 1 had been visualized is centrifuged at 8500 g for 10 min. An equal volume of 28% polyethylene glycol (average molecular weight 6000) solution is added to the supernatant. The resulting

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precipitate is centrifuged at low speed until the suspension is clarified.
The pellet is dissolved in tris buffer, sufficient ammonium
sulfate added to produce a concentration of 15% of satura-
tion, the resulting precipitate centrifuged until the sus-
5   pension is clarified, the pellet dissolved in tris buffer
and lyophilized or frozen for storage at -70^0C.

Example 3

A urine sample containing the togavirus-like particles is
purified by filtering the tris suspended pellet described in
10   Example 1 through a Amicon-filter membrane having an average
pore diameter of 0.8 microns, followed by ultrafiltration
with a Milipore PTHK membrane having a retention capability
for molecules of molecular weight greater than about 100,000.
The ultrafiltration is conducted at a slow rate so as to
15   minimize damage to the particles by fluid shear at the mem-
brane. After three volumes of tris buffer are passed
through the retentate, thereby washing the particles free of
lower molecular weight proteins, the retentate is ultra-
filtered to one-tenth of the applied sample volume, the mem-
20   brane briefly back-flushed with tris buffer, the retentate
recovered and lyophilized or frozen at -70^0C.

Example 4

Either the togavirus-like or enterovirus-like particles
may be radioiodinated by the following method. The particles
25   are suspended in phosphate buffered saline at pH 7.4. A stock
solution of ICl is prepared by diluting 0.56 g KI, 0.33 mg
$NaIO_3$, 29.2 g NaCl and 210 ml of concentrated HCl to 250 ml
with distilled water. Immediately before use, this stock
solution is extracted several times for 2 min. with 3 ml of
30   chloroform until the chloroform is no longer colored pink.
Residual chloroform is removed from the ICl stock by bubbling
water saturated air for 5 min. Finally, 1 ml of ICl stock
is thoroughly mixed with 10 ml of 2M NaCl. 0.1 ml of
particle suspension is diluted with an equal volume of 1 M
35   glycine buffer (pH 8.5) and mixed with 1 mCi of $Na^{125}I$ in 0.1
ml of the final ICl preparation and allowed to proceed for
5-10 seconds.   The product is rapidly separated from unre-
acted ^{125}I by gel filtration through a column (0.9 X 15 cm)
of Sephadex G-25 beads and eluted with phosphate buffered saline.

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Example 5

A vaccine may be made by adding formaldehyde to a purified suspension of either the togavirus-like or enterovirus-like particles (1 X 10^9/ml in phosphate buffered saline) to a final
5   concentration of 1:4000 and the mixture incubated with continuing agitation for 1 day at 37^0C and then 5 days at 4^0C. The reaction mixture is partially neutralized with sodium bisulfite to leave a final concentration of 10ug/ml formaldehyde. The vaccine is stored at 4^0C.

10   The vaccine is subcutaneously administered to experimental animals for 14 weeks to demonstrate the potency of the vaccine.

Example 6

For culture of either virus, the culture medium of Morgan et al., ("Proc. Soc. Exp. Biol. Med.", 73: 1 [1950]) is mod-
15   ified by adding 10% foal serum, 50 ug of dihydrostreptomycin/ ml and 100 units of benzylpenicillin/ml. The medium is sterilized by filtration through a 0.22 micron filter. An aliquot of the medium is placed into a plastic Falcon flask, followed by an inoculum of WI38 human diploid cells. The
20   inoculated medium is incubated at 37^0C until cellular confluence is achieved. A urine sample containing togavirus-like particles at a concentration of about 1 X 10^7 particles/ ml is added to the medium in a volumetric proportion of 1:75. The culture is incubated at 37^0C while maintaining the pH at
25   about 7.4 with dilute NaOH or HCl as appropriate. After about 7 days of incubation, cytotoxic effects on the tissue culture were noted, particularly conversion of the fibroblast cell line into substantially spherical, birefringent cells and, ultimately, cell lysis.

30   The particles are harvested from the culture by decanting the supernatant from the cell culture flask, centrifuging at low speed to remove cell debris, precipitating the virus by adding polyethylene glycol 6000 to a concentration of about 15% and centrifuging. The pellet is resuspended in a composition
35   consisting of one half part by volume of the culture medium without foal serum and one half part glycerol. The particles are stable in this composition when stored at -20^0C.

Example 7

The process of Example 6 is repeated with the following

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modifications: The inoculum is about 1×10^9/ml of ATCC
VR-2015 in a solution containing one half by volume of the
culture medium without foal serum and one half part glycerol.
The cell culture is a monolayer of MRC-5 diploid cells.

5   When the population of virus particles reaches about 1×10^9/ml
in the supernatant culture medium, accompanied by cytotoxic
changes in the cell monolayer, the culture is decanted,
centrifuged at 1500 X g to remove cell debris and harvested
by continuous ultracentrifugation. The population of re-
10 covered particles was adjusted to about 1×10^{11} particles/ml
by dilution into the suspending medium of Example 6.

Example 8

The purified togavirus-like particles or ATCC VR-2015 re-
covered in Examples 6 or 7 are emulsified in an equal volume
15 of Freund's complete adjuvant in accordance with known pro-
cedures. The antigen is then injected into the peritoneum of
a rabbit, followed by an intramuscular booster at 6 weeks.
After 14 weeks, serum is harvested from the animal and the
globulins precipitated with 40% ammonium sulfate. The pre-
20 cipitate is dissolved and dialyzed overnight against 200
volumes of 0.04 M phosphate buffer (pH 7.2).

Example 9

50ug aliquots of the antibody fraction of Example 8 are
radiolabeled with ^{125}I following Greenwood et al., "Biochem.
25 J." 89:114-123 (1963). The labelled IgG is separated from
free radioiodine by passage through a column of Sephadex G-50.
The labelled IgG is diluted to about 3 uCi/ml in a diluent of
50% calf serum, 5% human serum negative

for antibody to particle antigen and 0.1% NaN$_3$. This preparation is
stored at 4°C.

Example 10

The antibody to the enterovirus-like particle is purified by affinity
5   chromatography. The purified particle obtained in Example 7 is bound
to charcoal as follows: prewashed, sorbent carbon is packed into a
column. The particle suspension is introduced into the column at a flow
rate of approximately 200 ml per hour and recirculated until the column
is saturated, i.e., until no further decreases in particle titer occur
10   upon passage through the column. Residual nonspecific binding sites
are saturated by passing normal rabbit serum through the column, followed
by washing with 0.04 M phosphate buffer, pH 7.2 (PBS). Rabbit serum
is chosen because a rabbit is the animal immunized with the enterovirus-
like particle in Example 8. The collected rabbit serum is diluted into
15   PBS 1:2 and added to the immuno-adsorbent column at a flow rate of about
100 ml per hour. The column effluent is assayed for particle antibody
in accordance with the method of Example 13. Serum input is stopped
when particle-specific antibody begins to appear in the effluent.
The column is washed with PBS until the minimum effluent
20   protein content is reached as measured by ultraviolet adsorption. A
5 M NaI solution is passed through the column at about 100 ml per hour
to elute the antibody from the particles. The collected effluent is
ultrafiltered using an Amicon casette and 0.01 M sodium phosphate to
remove NaI and concentrate the solution. The solution is then filtered
25   through a 0.22 micron membrane and stored frozen at -70°C until used.

Example 11

The unlabelled antibody solution prepared in Example 10 may be label-
led with an enzyme. Many suitable, well-known methods exist for accom-
plishing such labelling. The following method, based on U.S. patent
30   Re. 29,169 is satisfactory:

5 mg of antibody and 20 mg of horseradish peroxidase (HRP) are
dissolved in 2 ml of 0.05 M phosphate buffer of pH 6.2. 40 μl of 25%
glutaraldehyde is added and the solution shaken at room temperature
for 2 hours. Then the mixture is dialyzed against PBS containing 0.001
35   Mg++ to remove glutaraldehyde, centrifuged at 250 xg, diluted in 0.05 M

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tris (hydroxymethyl) aminomethane buffer (pH 8), 1% normal human albumin
0.02% NaN$_3$ and 0.001 M MgCl$_2$. The residual unlinked enzyme may be re-
moved by gel chromatography in conventional fashion.

5   Other enzymes which may be coupled to the antibody include dehydro-
genases such as alcohol, glycerol, L-lactate, malate, glucose 6-phos-
phate and mannitol 1-phosphate dehydrogenase; oxidases such as glucose,
galactose, L- or D-amino acid, polyphenol or ascorbate oxidase; catalase;
cholinestease; alkaline phosphatase; B-glucuronidase; aldolase; and
histidase. Considerations in selecting the enzyme include cost, capacity
10   to be bound to the antibody, enzyme assay convenience, enzyme stability
and absence of the enzyme in the biological fluids to be assayed.

Example 12

       1 ml of unfractioned guinea pig serum obtained in substantially the
same fashion as described in Example 8 with respect to rabbits is coated
15   onto the inner surface of a polypropylene test tube in accordance with
U.S. Patent 3,646,346. 0.1 ml of a urine specimen from an acute NANB
hepatitis patient and 0.1 ml of a control specimen known to be free of
the particles are then placed into coated tubes and incubated for 12
hours. The unbound elements of the sample are washed from each tube with
20   water, labelled antibody of Examples 9 or 11 added to each tube and
incubated for 12 hours, unbound labelled antibody washed from the tube
with water and the radioactivity or peroxidase activity bound to the
tube determined. The particle antigens could be satisfactorily detected
by determining the extent of bound radioactivity or peroxidase activity
25   in the assay tube compared to the control tube. The procedure of this
Example is effective for either particle and for serum rather than urine
samples.

Example 13

       This method is representative of techniques for assaying anti-
30   viral antibodies. Solutions of   rabbit antihuman immune globulin
were prepared at 1:1000 in 0.01 M tris buffer, pH 9.0. Following the
procedure of U.S. patent 3,646,346, 1 ml aliquots of the solution
are placed into small, disposable polypropylene test tubes and allowed
to coat overnight. The tubes were then washed and air-dried.

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A serum sample and negative control is diluted 1,000 fold into 50% calf serum, 0.2% Tween-20 and 0.005 M EDTA in PBS. Duplicate 0.2 ml aliquots of the test samples are incubated overnight in the coated test tubes and then washed twice with 5 ml of water. 0.2 ml of the radiolabelled enterovirus-like particle made in accordance with Example 4 is added to each tube and incubated overnight. The label is decanted and the tubes are washed twice with 5 ml of water. The tubes are then counted in a gamma counter. The averaged count rates are compared with the negative control to determine whether or not the test sample is antibody positive.

If the test tube is initially coated with rabbit anti-human immune globulin which is specific for human IgG (prepared in known fashion), then the assay will be directed only to the gamma globulin portion of the test sample which is specific for the particle. Similarly, rabbit anti-IgM can be used to produce an assay specific for anti-particle IgM.

What is claimed is:

1. A method which comprises assaying a biological sample for ATCC VR-2015 antigen.

2. A method which comprises assaying a biological sample for anti-
5   body to ATCC VR-2015 antigen.

3. The method of claim 2 wherein the antibody is IgG.

4. The method of claim 2 wherein the antibody is IgM.

5. A method which comprises culturing ATCC VR-2015 in vitro in cell culture.

10 6. The method of claim 5 wherein the culture is WI-38 or MRC-5 diploid cells.

7. The method of claim 1 wherein the sample is assayed for said antigen by contacting the sample with water insoluble antibody to said antigen, removing the sample, contacting the insoluble antibody with
15 labelled antibody to said antigen, removing unbound labelled antibody from the insoluble antibody and assaying the label in either the bound or unbound fractions.

8. The method of claim 2 wherein the sample is assayed for antibody by contacting the sample with water insoluble specific binding
20 partner for the test sample antibody, contacting the insoluble antibody with a labelled ATCC VR-2015 antigen, removing the unbound specific binding partner from the insoluble antibody and assaying the label in either the bound or unbound fractions.

9. The method of claim 8 wherein the specific binding partner
25 is staphylococcal protein A or an antibody.

10. The method of claim 9 wherein the binding partner antibody is specific for IgG or IgM.

11. The method of claim 2 wherein the sample is assayed for said antibody by contacting the sample with water insoluble ATCC VR-2015 antigen and labelled antibody to said antigen, removing said sample and unbound labelled antibody from the insoluble antigen, and assaying the label in either the bound or unbound fractions.

12. A composition comprising an antigen of ATCC VR-2015 which is substantially free of primate proteins.

13. The composition of claim 12 wherein the antigen is a component of an intact ATCC VR-2015 particle.

14. A sterile composition comprising ATCC VR-2015 antigen in a pharmaceutically acceptable carrier.

15. The composition of claim 14 wherein the antigen is present as a component of biologically noninfectious ATCC VR-2015 particles.

16. The composition of claims 14 or 15 which is dry.

17. A composition comprising primate immunoglobulin which is substantially free of other primate proteins and contains antibody for binding an antigen of ATCC VR-2015.

18. A composition comprising nonprimate antibody for binding an antigen of ATCC VR-2015.

19. A composition comprising ATCC VR-2015 antigen bound to a detectable label or a water insoluble carrier.

20. The composition of claim 19 wherein the label is an enzyme, radioisotope, fluorescent group, luminescent group, coenzyme, enzyme modulator or stable free radical.
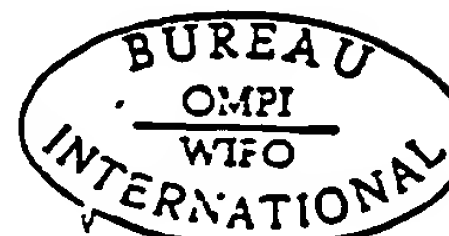
21. A composition comprising antibody to ATCC VR-2015 antigen which is bound to a detectable label or a water insoluble carrier.

22. The composition of claim 19 wherein the carrier is a polyolefin surface to which the antigen is adsorbed.

5   23. The composition of claim 21 wherein the carrier is a polyolefin surface to which the antibody is adsorbed.

24. A composition comprising sterile gamma globulin to ATCC VR-2015 antigen, which globulin is essentially free of antihemophilic factor and prothrombin complex.

10   25. The composition of claim 24 which is dry.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL. 435/7
INT. CL.$_3$ G01N 33/54

II. FIELDS SEARCHED

Minimum Documentation Searched	
Classification System	Classification Symbols
U.S.	435/7,68,188,240,241; 424/12,86,89,90

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

INDEX MEDICUS 1978-DATE, HEPATITIS, VIRAL, HUMAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with Indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	US,A, 4,196,265, PUBLISHED 01 APRIL 1980, KOPROWSKI ET AL.	1-4,7-11,17-25,
A	US,E, 29,169, PUBLISHED 05 APRIL 1977,SCHUURS ET AL.	1-4,7-11,11-23
X,P	US,A, 4,217,145, PUBLISHED 02 JUNE 1981,WANDS ET AL.	1-25
A	LANCET, 8217, 24 JANUARY 1981, SHU ET AL,"SPECIFICITY OF AN IMMUNOPRECEPITIN TEST...", PAGES 178-180.	1-25
A	LANCET, 8154, 15 DECEMBER 1979, VITVITSKI ET AL, "DETECTION OF VIRUS ASSOCIATED...", PAGES 1263-1267.	1-25
A	LANCET, 8095, 21 OCTOBER 1978, SHIRACHI ET AL, "HEPATITIS "C" ANTIGEN..."	1-25
A	LANCET, 8189, 09 AUGUST 1980, MORI ET AL, "VIRUS-LIKE PARTICLES...", PAGE 318.	1-25
A	SCIENCE, 205, 13 JULY 1979, SHIMIZU ET AL, "NON-A, NON-B HEPATITIS...", PAGES 197-200.	1-25
A	JOURNAL OF GENERAL VIROLOGY, 48, 1980, NEURATH ET AL, "AN ANTIGEN DETECTED...", PAGES 285-295.	1-25

 Special categories of cited documents:

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 MAY 1982	26 MAY 1982
International Searching Authority	Signature of Authorized Officer
ISA/US	JOHN E. TARCZA

Form PCT/ISA/210 (second sheet) (October 1977)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers_____, because they relate to subject matter [11] not required to be searched by this Authority, namely:

2.☐ Claim numbers_____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [12], specifically:

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

CLAIMS 1-4, 7-11 AND 19-23
CLAIMS 5-6 AND 12-16
CLAIMS 17, 18, 24, 25

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (October 1977)